A COMPREHENSIVE STUDY OF VIRUS INFECTING *CLERODENDRUM INERME* (L.) GAERTN EXHIBITING MOSAIC SYMPTOMS IN AGRA REGION

A SYNOPSIS

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INTRODUCTION

Plants have played an integral role in human society since the beginning of civilization. Genus *Clerodendrum* is a member of family *Lamiaceae* (earlier *Verbenaceae*) (Steane *et al*., 2004). The genus is widely distributed in tropical and subtropical regions and few sps. are found in the temperate regions too. The genus comprises of herbs, shrubs, and small plants. *Clerodendrum* is a very large and diverse genus and till now 580 sps. of the genus have been identified. Linnaeus in 1753 was the first to describe it as *C. infortunatum* (Shrivastava and Patel, 2007). The name "*Clerodendrum"* is derived from the Greek words for chance (klero) and tree (dendrum) and "fortunate" (Chance tree).

Clerodendum inerme (L.) Gaertn commonly known as garden quinine is a perenninal shrub grown as hedge plant in home gardens and parks. *C. inerme* is distributed in India, Sri Lanka, and South-east Asia. *C. inerme* is valued in landscaping as a bonsai, hedge plant or topiary. The plants are trimmed to form geometrical shapes and figures of birds and animals in the gardens. The plants are grown close together for a thick hedge look. Cattles normally avoid browsing them because of its bitter taste (Parmar *et al.*, 2013).

Botanical description of *C. inerme*

The plants of *C. inerme* are evergreen 1-1.8 mtr. tall sprawling shrubs having woody smooth stems. The morphology of leaves is ovate to elliptical (5-10 cm) long, acute to acuminate tip, green, smooth alongwith slightly shiny surface, pinnate venation, margins entire. Leaves are simple and opposite. Cyme usually comprised of 3 flowers joined at a common base point, corolla white, fused, with 5 lobes, 4 stamens, reddish to purple and upwardly curved. Fruit is 1-1.5 cm long obovoid (Chethana *et al*., 2013).

Chemical constituents

Chemically the genus is very rich in phenolics, steroids (*β*-sitosterol, *γ*-sitosteroloctacosanol, clerosterol, bungein A, acteoside, betulinic acid, clerosterol), di- and triterpenes, terpenes such as α-amyrin, *β*-amyrin, caryoptin, 3-epicaryoptin, clerodendrin A, B and C, 16-hydroxy epicaryoptin, clerodin, cleroinermin, clerodermic acid etc. (Shrivastava and Patel, 2007).

SIGNIFICANCE

Medicinal plants play a key role in health promotion of world. Venkatanarasimman *et al* (2012) estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants.

Apart from being a popular hedge plant, ethno-medicinal importance of *C. inerme* has been reported in folk medicines and in various indigenous systems of medicines (Shrivastava and patel, 2007). According to Sasikala *et al* (1995), *Clerodendrum* is used under the names of Chankankuppi and Pechagnan, in Siddha medicine.

C. inerme is potent to treat serofulous infection, coughs, venereal infections skin diseases, buboes problem, and as a febrifuge, vermifuge and also for treatment of Beriberi disease and is also used by tribals as an antidote of poisoning from toads, crabs and fish (Rehman *et al*. 1997, Kanchanapoom *et al*. 2001, Pandey *et al*. 2003).

Ravindran *et al.* (2005) reported that leaves of *C. inerme* are used for removing pain and in jaundice. Paste and leaf extract are used in treatment of malaria, inflammation, itching skin diseases and infected wounds.

Rahmatullah *et al* (2011) reported that tribal medical practioners of Bangladesh used *C. inerme* leaves and flowers for the treatment of pneumonia, rheumatoid arthritis, night blindness and skin diseases.

Sinha *et al* (2013) used *C. inerme* leaf paste about 2-3weeks for treatment of scabies and

leucorrhoea.

Apart from being ethno medicinally important, *C. inerme*, is also experimentally valuable as Sangeetha *et al* (2011) used *C. inerme* leaves to study in vitro anti-inflammatory and anti arthritic activity, Chahal *et al* (2010) used crude extract of *C. inerme* to inhibit the activity of some human pathogenic microorganisms- *Bacillus subtilis, Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* and fungal strains of *Aspergillus niger, Aspergillus flavus, Candida albicans* and *Candida glabrata.* Thus it can be exploited as future natural plant based antimicrobial agent. Chakraborthy *et al* (2013) reviewed antimalarial, antioxidant, antihepatoprotective, antiviral activity against Hepatitis B virus, anti heamolytic, antihypotensive, and antifungal activities of *C. inerme.*

SELECTION OF TOPIC

Now a days viruses are responsible for a number of diseases in different plants. Plant viruses are causing severe losses and extensive damage to the plants. There are few reports of viral diseases affecting plants of this genus (John *et al*., 2006). Chlorotic to necrotic spots on bleeding heart leaves were first observed in a residential garden at Piracicaba associated with infestation by *Bravipalpus phoenicis.* Infestation of mites collected from symptomatic plants revealing that an N-BTV was the causal agent, named *Clerodendrum* chlorotic spot virus (ClCSV) (Kitajima *et al.*, 2008).

A yellow mosaic of *C. inerme*, caused by a begomovirus was reported in India (John *et al*., 2006) and Schuta *et al*. (1997) found association of an unidentified rhabdovirus in C. *speciosum* showing vein clearing (Kitajima 2008).

Naturally infected *C. thomsoniae* leaves showed characteristic ring-spot symptoms consisting of definite alternating bands of chlorotic yellow and green rings, necrotic rings, puckering, chlorosis and distortion of young leaves were most pronounced during summer and fall (Khan and Maxwell, 1975).India is a large country with very diverse agro-climatic conditions which makes

it ideal for plant viruses (Paul *et al.* 2012).

A survey was conducted during January 2013 t o August 2013 in some parks and gardens of north east areas of Agra region, for the visual inspection of disease symptoms like chlorotic spots, mosaic, mottling, puckering, inward rolling of leaves etc. on the *Clerodendrum inerme* plant hedges. Approx. **77.33 %** of *C. inerme* plants were found naturally infected. A wide variety of symptoms observed on the indicator plants ranged from chlorotic spots to puckering, mosaic, vein clearing, irregular yellow and green patches on leaves, reduction in leaf size, stunting in plant growth, complete yellowing of apical leaves. Total infection on host plants was 65.21%, when host plants were inoculated with 0.1 M sodium phosphate buffer, and 76.66%, when host plants were inoculated with 0.5 M sodium borate buffer. Chip grafting was successful for the transmission of disease agent when healthy plants of *C. inerme* were grafted with symptomatic plants of *C. inerme*. The preparation under study showed the presence of isometric particles, 50-60 nm in size through electron microscopy. Infectivity assays and electron microscopy confirmed the viral nature of the disease agent (Singh and Gupta 2013).

As per literature reviewed, the isolated virus is different from all the previously reported *C. inerme* associated viruses in the mode of transmission, morphology, shape and size. All workers have mostly reported begomovirus in *Clerodendrum inerme* plants, which is a geminate particle (dumble shaped in morphology) and cannot be mechanically transmitted but the isolated virus particles under present study were spherical in shape, and mechanical transmission was also successful.

Present study aims to purify this rather new virus and characterize its viral nucleic acid and proteins, along with some studies.

OBJECTIVES

The study will aim to investigate the *Clerodendrum* mosaic associated virus in details with the following objectives:

1. To study disease prevalence of *Clerodendrum inerme* yellow mosaic in Agra region

2. To further study biological properties of virus to complete its biological characterization.

3. To study few intrinsic physico-chemical and electron microscopic properties through purification and characterization of virus from symptomatic leaves.

4. To further study effect of virus on plant host physiology.

5. To control virus incidence using some plant extracts.

REVIEW OF LITERATURE

Burnett and Youtsey (1962) reported a pathogen from *C. thomsoniae* with zonate ring spot. Symptoms to healthy plants were transmitted by means of leaf grafting. They were unable to transmit the pathogen by sap inoculation techniques.

Khan and Maxwell (1975**)** identified tobacco ringspot virus in *Clerodendrum thomsoniae*. The causal agent was identified as an isolate of Tobacco Ringspot Virus because of its host range, physical properties, particle morphology and serological relationships. This was the first identification of a virus disease of *Clerodendrum sps.*

Childers *et al*. (2001**)** reported *Clerodendron* chlorotic spot and *Clerodendron* green spot diseases transmitted by *Brevipaplpus* mites in Brazil, in which *B. Pheonicis Clerodendron* chlorotic spot transmitted.

Childers *et al*. **(**2003**)** recorded *Clerodendron fragrans* Ventenat, *Clerodendrum fallax* Lindley, *Clerodendron inerme* R. Brown, *Clerodendron indicum* Kuntz, *Clerodendron paniculatum* L., *Clerodendron siphonanthus* R. Brown, *Clerodendron speciosum* Gürke, *Clerodendron speciosissimum* Paxton, *Clerodendron serratum* Moon, *Clerodendron splendens* G. Don, *Clerodendrum Wallichii* Merrill, *Clerodendron thomsoniae* Balfour, *Clerodendrum* sp. along with as host plant species of *Brevipalpus californicus*, *B. Phoenicis* and *B. obovatus*, involved in the spread of viral diseases.

Kitajima *et al*. (2003**)** identified *Brevipalpus* transmitted plant virus and virus like diseases that affect economic plants including *Clerodendrum*. Green spot symptoms have been associated with the cytoplasmic type of all alternations in *Clerodendrum*.

John *et al*. (2005**)** reported a new begomovirus found associated with yellow mosaic disease of *Clerodendron inerme*. They tested symptomatic leaves and apparently leaves without symptoms for virus infection by using southern hybridization, polymerase chain reaction (PCR)-mediated amplification, and electron microscopy (EM). Total DNA extracted from 15 symptomatic samples showed positive hybridization to a radioactively-labeled tomato leaf curl New Delhi virus-[potato] DNA A probe, indicating the presence of a begomovirus. No characteristic virus particles were detected by EM. No hybridisation was detected in samples extracted from plants without symptoms. The virus associated with yellow mosaic disease of *Clerodendron* is a previously unidentified begomovirus, indicated by the low levels of sequence identity

Kitajima (2007) proposed a research project dealing with several plant viruses transmitted by the tenuipalpidae mite *Brevipalpus* spp. and intended to give a significant advance on the knowledge of the virus/vector/host plant relationships including pathological, biochemical, biological, morphological, molecular, cytopathological immunological aspects as well as resolving problems in epidemiologic, taxonomic and management of the disease/vector studies. The final goal was to get epidemiological parameters which may lead to a more efficient and less expensive control measure of diseases economically important as citrus leprosis and passion fruit green spot.

Jose *et al*. (2008) carried out a survey for the identification of *Brevipalpus*-associated plant viruses, in Brazil, characteristics considered were the occurrence of localized symptoms, reporting cases of infection by putative BTrV of chlorotic ringspot and green spot of cytoplasmic type in *Clerodendrum speciosissimum.* The putative nuclear type of BTrV found in bleeding heart was probably previously described as *Clerodendrum* chlorotic spot virus (CICSV).

Kitajima *et al*. (2008) found chlorotic spots on *Clerodendrum* which was a disease caused by a nuclear type of *Brevipalpus* transmitted virus, infesting symptomatic *C. x speciosum* plants. The

pathogen was transmitted to healthy *C. x speciosum* and to *C. thomsoniae* causing chlorotic spots on their leaves. The virus was purified from systemically infected leaves of *C. amaranticolor* and *C. quinoa*. A polyclonal antiserum obtained from an immunized rabbit presented a strong reaction with the homologous antigen in ELISA tests. The results suggested that this chlorotic spot disease of *C. speciosum* is caused by a new species of the nuclear type of BTrV, tentatively named *Clerodendrum* chlorotic spot virus *(ClCSV)*.

Yang *et al*. (2009) reported *Clerodendrum Yellow Mosaic China* Virus isolated from *Clerodendrum cyrtophyllum*. An isolate denoted Fz7 was selected for further sequence analysis. DNA-A of Fz7 was most closely related to that of an isolate of *Clerodendron* golden mosaic virus (CIGMV8VN: Son: 05, Gen bank accession no. DQ1641692) with 78.9% nucleotide sequence identity. Fz7 appeared to be distinct bipartite begomovirus for which the name *Clerodendrum* Yellow Mosaic China Virus had been proposed. This was first report of a bipartite begomovirus infecting *Clerodendrum cyrtophyllum* in China.

Kubo *et al*. (2009) evaluated the genetic variability of orchid fleck virus by single-strand conformational polymorphism analysis and nucleotide sequencing of a fragment from the nucleocapsid gene and reported that *Clerodendrum* chlorotic virus (ClRSV) had genomes partially sequenced revealing some similarity with that of OFV.

Chatterjee *et al*. (2009) reported CAPS analysis as a possible tool to detect and group Gemini viruses infecting some fibre crops and weeds. In their study, the grouping of begomoviruses based on CAPS analysis revealed that the isolates obtained from the same family *Malvaceae* shared a common phylogenetic relationship, which appeared to be in line with the fact that the satellite DNA β molecule of begomoviruses showed greater resemblance among linked hosts than with begomoviruses infecting distantly related hosts alongwith *Clerodendrum indicum* with yellowing

in leaf. The present reading indicated that the CAPS technique might be a very helpful implement for identification and grouping of begomoviruses especially when sequence information is not available.

Karyna *et al*. (2009) reported that reclaimed water which is an important component of sustainable water resource management was regarded as pathogen transport through this alternative water supply. This study characterized the viral community found in reclaimed water and compared it with viruses in potable water. Reclaimed water contained 1000-fold more viruses like particles than potable water, having approximately 108 VLPs per milliliter. Reclaimed water may play a role in the dissemination of highly stable viruses. Information regarding viruses including *Clerodendron yellow mosaic virus* present in reclaimed water but not in potable water can be used to identify new bio indicators of water quality.

Ding *et al.* (2010) reported the occurrence of *Clerodendrum* golden mosaic virus in *Clerodendrum bungei* in Yunnan in China*,* in 2009, a virus isolate (YN1095) was recognized and characterized from *C. bungei* plants showing chlorosis and yellow mosaic in Jinghong municipality (Yunnan Province, China).

Kitajima *et al.* **(**2010) reported the first cases of ornamental plants infected by *Brevipalpus* transmitted viruses (BTV). These were described in 1990‟s from the region of Piracicaba, State of São Paulo, Brazil, subsequent cases were from other regions in the country and other American countries. Currently, 37 ornamental plant species belonging to 18 families of dicotyledons have been reported hosting BTV.

Li and Zhou (2010) reported two begomovirus isolates (YX2-I and YX2-II) from *Clerodendrum cyrtophyllum* showing yellow mosaic symptoms collected in Jiangsu province of China. Sequence analysis revealed that YX2-I is a distinct begomovirus species for which the name *Clerodendrum*

golden mosaic Jiangsu virus (ClGMJSV-[CN: YX2:08]) is proposed. YX2-II is an isolate of bipartite begomovirus *Clerodendrum* golden mosaic China virus (ClGMCNV- [CN:YX2:08]).

Sivalingam *et al.* (2010) reported that the complete genome (DNA A) of *Clerodendron yellow mosaic virus* was cloned and sequenced. The total size of DNA A is 2760 nucleotides. The genome of this virus contains six open reading frames and a non-coding intergenic region of 293 nucleotides.

Nucleotide sequence comparison analysis revealed maximum sequence identity with Papaya leaf curl virus-Pakistan [Pakistan: Cotton: 2002] (73.9%). As this virus had less than 89% identity with other begomoviruses, it was identified as a new begomovirus species and tentatively, named as *Clerodendron* yellow mosaic virus-[India: New Delhi: 2007] (ClYMV-[IN:ND:07]).

Paul *et al.* (2011) analyzed coat protein gene sequences of begomoviruses associated with different weed species including *Clerodendron inerme* L., in India. They are often infected by different begomovirus complexes and may act as reservoirs of crop-infecting begomoviruses. Results revealed that the weed infecting begomovirus complexes were geographically highly diverse among themselves throughout India,as indicated in the case of *Sida*, *Eclipta*, *Croton* and *Clerodendrum*, where geographically separated isolates had a low sequence similarity score (%) among themselves.

Kubo *et al.* (2011) confirmed the presence of *Brevipalpus*-transmitted virus in their mite vectors by RT-PCR. The identification of plant diseases caused by *Brevipalpus*-transmitted viruses (BrTVs) had been done through the analysis of symptoms, transmission electron microscopy, and RT–PCR of infected plant tissues. The detection of *Citrus* leprosis virus C, Orchid fleck virus, *Clerodendrum* chlorotic spot virus and *Solanum violaefolium* ringspot virus in their viruliferous vectors *Brevipalpus* spp. using specific primer pairs for each of the viruses.

Valverde *et al*. (2012) detected and identified **"***Clerodendron* Golden Mosaic China Virus" in *Salvia splendens,* by obtaining two viral DNA"s from *Salvia splendens* showing variegated foliage similar to virus symptoms. Sequence analyses revealed the presence of an isolate of the putative begomovirus *Clerodendron* Golden Mosaic China Virus. The virus was detected in all *Salvia* plants showing variegated foliage, but not in non-variegated plants. Attempts to transmit the virus with the white-fly *Bemisia tabacci* biotype B failed. Results of their investigation strongly suggested that *Salvia* isolate of *Clerodendron* Golden Mosaic China Virus (ClGMCNV-Sal [USA: LA: 11]) was the cause of the variegated foliage of *Salvia splendens* cv. (dancing flame). This was the first report of this virus in the United States.

Anwar *et al.* (2012) gave first report of *Clerodendron* Yellow Mosaic Virus infecting Croton. Leaf samples from two croton plants, showing mild leaf curl and yellowing symptoms, and from two symptomless plants were collected in Lahore (Pakistan) during 2012. DNA was extracted from samples by the CTAB method. Begomovirus was confirmed by amplification of a product of the expected dimension (*ca.* 2800 nts) in PCR with adjoining primers BGAF/BGAR, from symptomatic but not from non-symptomatic leaf samples. The sequence indicated 99.1% nucleotide sequence identity to *Clerodendron* Yellow Mosaic Virus (ClYMV, accession No. EF408037) and thus represents a new variant of ClYMV. Attempts to identify the presence of a second component using primer specific for DNA B BGBF/BGBR and Beta01/02 primers for betasatellites failed to produce an amplification product. Since Croton is a widely grown evergreen plant it may harbor begomoviruses for many years and act as source of inoculum for crops. This was the first report of ClYMV occurring in Pakistan and the first time that this virus had been identified in Croton.

Valerio *et al.* 2012 analyzed sample of *Clerodendrum sp.* along with 40 leaf samples for the

presence of geminiviral DNA growing present in the cotton growing areas of North Western India.

METHODOLOGY

Maintenance of virus culture

Sap extracted from the symptomatic leaves of *C. inerme* will be inoculated to test plants. Test plants showing best systemic symptoms will be used for maintenance of virus culture, along with symptomatic *C. inerme* which will be used as stock culture.

1. TO STUDY DISEASE PREVALENCE OF *CLERODENDRUM INERME* YELLOW MOSAIC IN AGRA REGION

Number of restricted surveys will be conducted from March 2015 to February 2016 in various areas of Agra region, to identify presence of *C. inerme mosaic* symptoms. *C. inerme* plants showing vein clearing, leaf roll, mosaic, chlorosis, necrosis, yellowing and leaf deformity will be visually inspected.

Symptomatic leaves of *C. inerme* will be collected randomly from different locations. Disease incidence and severity will be calculated separately for each survey as follows:

$$\text{Disease incidence} = \frac{\text{No. of infected plants per location} \times 100}{\text{Total no. of plants per location}}$$

$$\text{Disease severity} = \frac{\text{Area of plant tissue affected by disease} \times 100}{\text{Total area of leaf}}$$

2. TO COMPLETE BIOLOGICAL CHARACTERIZATION OF THE VIRUS

Viral nature of the symptomatic *C. inerme* was successfully established by mechanical transmission method. Few plants belonging to families *Amaranthaceae*, *Cucurbitaceae*, *Leguminoseae*, *Cucurbitaceae*, *Amaranthaceae*, *Solanaceae* and *Euphorbiaceae* were successfully mechanically inoculated. The families and plants not used earlier for transmission will be included for transmission to widen the host range studies and expression of symptoms, therein.

2.1) Mechanical transmission studies

2-3 test plants of each species will be inoculated with the extract obtained from chosen indicator plant. The test plants or host plants at 2-3 leaves will be inoculated on the upper surface of the leaves with the extract, using celite powder as abrasive. Inoculations will be unidirectional with light hand. After inoculation, the leaves will be rinsed with distilled water to remove superfluous inoculum and kept in green-house. The uninoculated /healthy plants will be maintained as control plants. Inoculated and control plants will be observed daily for the development of any visible symptoms.

2.2) Graft transmission studies

2.2.1) Chip grafting

Chip grafting was successful to establish union previously; using a bark chip (2.5-3.0 cm) from the smooth surface of internodes of stock plant. Now the cleft graft method will be used, in present study.

2.2.2) Cleft grafting

A graft transmission study will be conducted in accordance with Shah H. *et al.*, 2008. Wedge grafting will be used to test the transmissibility of viral infection of indicator plant showing

mosaic and chlorotic spots. Healthy plants of the different species will be chosen as recipient or rootstock. The scion from the symptomatic plants will be taken and a wedge will be made with a sterile sharp scalpel knife by removing cambium tissue. A slanting cut of about 5mm will be made in the rootstocks of healthy plant species and wedge shaped cut on scions will be inserted. Adhesive tape will be tightly wrapped around the insertion portion. Similarly healthy plants will be auto-grafted as negative check. The grafted plants will be covered in moist polyethylene bags, well irrigated and placed in shade for one week in insect-proof cages under growth room conditions till establishment union. Insecticide will be applied to discourage insect feeding. After complete union formation, the plants will be shifted to glasshouse and will be observed for symptoms development. About five plants of each host will be grafted.

2.3) Vector transmission studies

Observation studies for any insect visitor on *C. inerme* will be done along with the survey. Any insect visitor at different time period of the day will be collected, identified and attempts will be made for transmission. In addition, aphids and whiteflies will be collected from fields and vector transmission will also be attempted.

2.3.1) Aphid transmission

Aphids will be collected from the mustard field in winter season, using a yellow colour cloth and will be transferred to the petridish. Aphids will be stored in a cool shaded place for about 1 hour to starve them. Aphids will be transferred to symptomatic plant material (detached leaves in a separate Petri dish) to feed for about two minutes (Acquisition feeding). At least five aphids will be transferred to separate indicator plant. Setup will be covered to contain aphids and allow an inoculation feed of approx. 1 hour. Aphids will be killed by spraying indicator plant with insecticide solution and test plants will be set aside in glass house to grow on for symptom observation (Hill S. A., 1984).

2.3.2) Whitefly transmission

Whiteflies will be collected from the leaves of cucurbitaceous plants as *Lageneria siceraria*, *Cucumis sativus, Cucumis melo* etc., by using test-tube. The test tube will be placed on the ventral surface of the leaf. Whiteflies will move up in the test-tubes towards the direction of light. Test tubes containing whiteflies will be plugged with cotton plug. These whiteflies will be given a fasting period, of about an hour. Starved whiteflies will be then transferred to the symptomatic plant material (detached leaves of *C. inerme* in a separate Petri dish) to feed for about two minutes for acquisition feeding. At least five whiteflies will be transferred to each separate indicator plant. Cover to contain whiteflies and allow an inoculation feed of approx. 1 hour. Whiteflies will be killed by spraying indicator plant with insecticide and plants will be set aside in green house to grow on for symptoms observation (Hill S. A., 1984).

Persistence of infectivity in extracted sap will be checked after stepwise dilution (dilution end point), storage at room temperature (longevity in vitro) and heating in vitro (thermal inactivation point) for 10 minute.

3. TO STUDY FEW INTRINSIC PHYSICO-CHEMICAL AND ELECTRON MICROSCOPIC PROPERTIES THROUGH PURIFICATION AND CHARACTERIZATION OF VIRUS FROM SYMPTOMATIC LEAVES.

3.1) Optimization of buffer and pH

To obtain purified concentrated virus preparations, several buffers such as phosphate, borate, acetate and Tris buffer will be used with molarities ranging from 0.01- 0.1 adjusted with different pH 6.5-8. The buffer with suitable molarity and pH will be used for the purification of virus.

3.2) Concentration and purification of virus

3.2.1) Protocol-1: Acidification of plant extract of *C. inerme*

The symptomatic leaves of indicator plants will be purified by slight modifications of the methods followed by Stace Smith *et al*. (1965), Steere (1956) and Kitajima (2008). Fresh symptomatic leaves will be fragmented with suitable buffer (1 ml/ gram leaf tissue) containing 20 mM 2-merceptoethanol as a stabilizer. The fragmented tissue will be squeezed through cheesecloth, clarified with an equal volume of n-butanol: chloroform mixture (1:2 v/v), and stored for 6 to 8 hours. The denatured protein will be removed by low speed centrifugation (10,000 rpm for 10 minutes) and the virus will be then pelleted by adding acetic acid by high speed centrifugation for 2 hours. The resulting pellets will be resuspended in 10 mM ethylenediaminetetraacetate (EDTA), pH 7.0, and clarified by low speed centrifugation. The purified virus preparation will be obtained with the polyethylene glycol precipitation procedure. Polyethylene glycol and Nacl will be added with stirring to the clarified to final concentrations of 10 % and 0.3 M, respectively. The mixture will be allowed to stand for one hour at 4 ºC, and then the precipitated virus will be pelleted by a low speed centrifugation (10,000 rpm for 10 minutes). Optical density of purified sap and supernatant will be noted using spectrophotometer at 230 -290 nm.

3.2.2) Protocol 2: Salt precipitation method

The purification method of Nerway and Kassim (2014) with slight modifications will be used for the purification of virus from indicator plant.

In extraction process, 200 g leaves of the indicator plant, which kept frozen, will be mixed with suitable buffer. The mixture will be grounded with blendor machine for 3-4 minutes. Suspension will be collected. Then 0.1M ascorbic acid and EDTA will be added to the suspension.

In clarification process, 40% of the chloroform will be added to the supernatant with minutes strong mixing using blender machine. Prepared suspension will be poured into centrifuge tubes

and spun at 10000 rpm at 4 ^{0}C for 20 minutes in a cooling centrifuge. Supernatant will be collected and placed in an icebox. Ammonium nitrate will be added to the half saturated point. Centrifuge tubes will be filled with suspension and spun at 14000 rpm for 2 hours. The pellet will be taken and resuspended in buffer. Optical density of purified sap and supernatant will be noted using spectrophotometer at 230 -290 nm.

3.3) Confirmation of purified virus preparation through Electron microscopy

The sap (supernatant and pellet dissolved in 30mM potassium phosphate buffer obtained from both the methods (alcohol precipitation and salt precipitation) will be applied to carbon coated grids, stained with uranyl acetate and rinsed with distilled water. Concentrated and purified preparation of virus will be checked by examining grids under Transmission Electron Microscope, and particle shape, size, no. of particle types etc. will be observed.

3.4) Characterization of virus

Ultra-violet absorption and A_{260}/A_{280} will be calculated. Characterization of proteins and nucleic acid will be done through SDS-PAGE (Sodiumdodecyl Polyacralamide gel electrophoresis) and AGE (Agarose gel electrophoresis).

Gel electrophoresis

2.4) Stock acrylamide solution

Acrylamide 30%

Bisacrylamide 0.8% and distilled water

2.5) Separating gel buffer

1.85 M Tris Hcl buffer pH 8.8

c) Stacking gel buffer

0.6 M Tris Hcl buffer pH 6.8

d) Polymerizing agents

Ammonium persulphate 5%

TEMED- fresh from refrigerator

Acrylamide solution will be made up, for separating gel and casted in vertical slabs. The solution will be carefully overlaid with isobutanol saturated with water to avoid inhibition of polymerization. Laemmelli buffer will be used for characterization of proteins and TAE or TBE, for nucleic acids. After the polymerization of the separating gel, the saturated isobutanol will be substituted with the stacking gel (5%), then the appropriate comb will be inserted and the gel will leave to be polymerized. The slab will be electrophoresed for 3-4 hour till the marker dye bromophenol blue (BPB) reach the end of the gels. The gel will be fixed in 12 % trichloroacetic acid for 1 hours and stained with coomassie blue/ silver nitrate for proteins and EtBr for nucleic acid. After 3-4 hours, gel will be scanned in transilluminator (Sadasivam, 2005).

4) TO FURTHER STUDY EFFECT OF VIRUS ON HOST PLANT PHYSIOLOGY.

Effect of virus on carbohydrate contents viz. sugars, starch, primary productivity, and pigment contents was attempted earlier in which in diseased leaves, reducing sugars and starch content were less than the healthy leaves, and non- reducing sugar contents were more than the healthy leaves. The capacity of photosynthesis of symptomatic leaves was much reduced than the healthy leaves and respiration was increased in symptomatic leaves than the healthy leaves. These results illustrated a general loss of chlorophylls and carotenoids in symptomatic leaves due to virus infection. Further study will cover other physiological parameters as amino acids, proteins,

nitrogen content and enzymatic activity.

4.1) Free amino acids

Two lots of plants will be taken. One lot of plants will be inoculated with *Clerodendrum inerme* associated mosaic virus and the second lot will be kept as control. Free amino acid content of leaf, stem and root from the diseased and healthy plants will be estimated after 5, 10, 15, 20 and 30 days of inoculation. Free amino acid content will be estimated the method described by Moore and Stein (1948), Sadashivam (1996).

500 mg of plant material will be taken and grind it in a pestle mortar with a small quantity of acid washed sand. 10 ml of 80 % of ethanol will be added to the homogenate. Homogenate will be centrifuged. Supernatant will be collected. Extraction will be repeated twice with the residue. To the 0.1 ml of extract, 1ml of ninhydrin solution will be added. Volume will be make up to 2 ml with distilled water. Tube will be placed in waterbath for 20 minutes. 5 ml of diluent solvent (eqaul volumes of water and propanol) will be added. After 15 minutes, the intensity of purple colour will be read against a reagent blank at 570 nm.colour will be atble for 1 hour. Reagant blank will be prepared by taking 0.1 ml of 80 % ethanol instead of extract. A standard solution will be prepared; 50 mg luecine will be dissolved in 50 ml of distilled water in a volumetric flask. Standard curve will be prepared using absorbance and concentration.

4.2) Protein

In this experiment two lots of plants will be taken. The first lot of plants will be inoculated with sap of *C. inerme* associated mosaic virus and second lot will be kept as control. Protein content of leaf, stem and root of healthy plants will be estimated separately after 5, 10, 15, 20 and 30 days of inoculation. Protein content will be determined using a spectrophotometer procedure as per Lowry *et al* (1951), Sadasivam (2005).

5 gms of dried plant material (leaf, stem and root) will be crushed with 10 ml of 20 %

trichloroacetic acid. Homogenate will be centrifuged at 6000 rpm for 15 min., the supernatant will be discarded. To the residue, 5ml of 0.1N NaOH will be added and centrifuged. The supernatant will be collected and made to 5ml with 0.1N NaOH. From the extract, 0.5 ml of the sample will be taken in a testtube and 5 ml of alkaline copper solution will be added. The solution will be mixed well and made to stand in darkness for 10 min. 0.5 ml of folin-phenol will be added with vigorous mixing. The mixture will be kept in dark for 30 min. the sample will be read at 660 nm in spectrophotometer. Blank will be prepared without protein sample. Standard graph of protein will be prepared by using 5^{th} fraction of Bovin Serum Albumin. The amount of protein will be determined by multiplying its Kjeldahl nitrogen content by a factor of 6.25. The amount of protein will be expressed as mg/g.

4.3) Total nitrogen

In this study two lots of plants will be taken. The first lot of plants will be inoculated with *C. inerme* associated mosaic virus and the second lot will be kept as control. Total nitrogen of leaf, stem and root from symptomatic and healthy plants will be estimated separately after 5, 10, 15, 20, 30 and 40 days of inoculation. Total nitrogen will be estimated by method followed by Magomya *et al* (2014). In each treatment the material to be treated will be taken from five plants. This material will be dried at room temperature for 72 hours.

2 g of powdered sample will be digested in a Kjeldahl digestion flask by boiling with 20 ml of concentrated H_2SO_4 and a Kjeldahl digestion tablet (catalyst) until the mixture was clear. The digest will be filtered into a 250 ml volumetric flask and the solution will make up to mark with distilled water and connected for distillation. Ammonia will be steam distilled from of the digest to which 50 ml of 45% sodium hydroxide solution will be added. 150ml of the distillate will be collected in a conical flask containing 100ml 0.1N HCl and methyl red indicator. The ammonia that distilled into the receiving conical flask react with the acid and the excess acid in the flask will be estimated by back titration against 2.0M NaOH with colour change from red to yellow

(end point). Determinations will be made on all reagents alone (blank determinations).

% Nitrogen was calculated as follows:

$$= \frac{\text{(ml standard acid x N of acid)-(ml blank x N of base) x 1.4007}}{\text{Weight of sample in grams}}$$

Where N=normality

4.4) Enzymatic activities

In plants, measurement of several enzyme activities have been made on normal and virus infected tissues by various workers, and have found changes in enzyme activities.. In this study, effect of *Clerodendrum* associated yellow mosaic virus infection on peroxidase and catalase activities in suitable host plant will be made.

4.4.1) Peroxidase activity

One lot of plant will be inoculated with the virus and the second lot will be kept as control. This will be measured by the method of Perur (1962). Crude enzyme will be extracted from 1 g of plant material (leaf, stem and root) by crushing it in a chilled mortar with a pinch of acid with acid washed sand and 5 ml of ice cooled glass distilled water. The homogenate will be made to 50 ml. It will be filtered through four folds of cheese cloth. This filtrate will be the crude enzyme preparation used for the measurement of peroxidase activity. Identical enzyme preparations will be made separately from healthy and infected plant material. In a colorimetric tube, 10 ml of acetate buffer (pH 4.5), 1 ml of crude enzyme and 0.5 ml of 1% pyrogallol solution will be added and mixed thoroughly. At zero time, 0.5 ml of 0.05N H_2O_2 will be added to the mixture and immediately the contents will be mixed. Change in optical density of the mixture at 430 mμ in absorptiometer will be measured.

4.4.2) Catalase activity

Measurement of catalse activity will be made according to the perborate method of Feinstein (1949) as described by Dekock et al. (1960) with slight modifications. For enzyme preparations, 1 gm of plant tissue (leaf, stem and root) will be crushed in a mortar with 10 ml of 0.1M phosphate buffer (pH 6.8), the homogenate will be transferred to a 25 ml volumetric flask along with two washings with distilled water. The volume will be made to 25 ml with addition of distilled water and then filtered through cheesecloth. This will be done separately with healthy and diseased samples. A series of flasks containing 5 ml of 1.5 % sodium perborate and 1.5 ml of 0.1M phosphate buffer (pH 6.8) will be arranged. Aliquots of 1 ml enzyme extract will be blown into each flask and the reaction will be stopped in successive flasks after 1, 2, 3 and 4 minutes by rapidly adding 10 ml of 1N H_2SO_4. The remaining perborate in the flask will be titrated with 0.05N $KMnO_4$ to the first pink colour which lasted for 30 seconds. The quantity of $KMnO_4$ consumed will be recorded and activity of catalase will be calculated as follows:

$r = 4Y4+3Y3-Y2-6Y1 / 4Y3+3Y2-Y1-6Y0$

Where Y0, Y1…Y4 are values of $KMNO_4$ used for the flasks in which the reaction will be arrested at 0, 1, 4 minutes respectively.

$K= -\log r10$

where K= constant

$A=K/W$

Where A= activity in units/g fresh weight

W= fresh weight of the plant material in grams.

5) TO CONTROL VIRUS INCIDENCE USING SOME PLANTS EXTRACTS

The methodology for inducing systemic resistance used by Verma *et al* (1996) will be employed. Leaf extract of *Mirabilis jalapa* and root extract of *Boerhavia diffusa* will be used for induction of systemic resistance against *Clerodendrum* associated mosaic virus.

Preparation of virus inoculum

Culture of *Clerodendrum* associated mosaic virus will be maintained by regular passes in systemic host. Virus inoculum will be prepared by grinding 5 gm fresh leaves of of diseased plant with distilled water. The pulp will be squeezed through two layers of muslin cloth and the filtrate will be centrifuged at 5000 rpm for 15 minutes. The supernatant will be diluted with distilled water to obtain no. of local lesions on leaves after inoculation with virus inoculums.

Host plants

Plants that show systemic infection through mechanical transmission will be used as host plants. For experimental work, plants will be used at 5-6 leaves stage. All experiments will be performed with a minimum of three replicates (minimum three plants with three to four leaves) per treatment.

Assay for virus inhibition

The upper two leaves of host plants will be inoculated with obtained extract of chosen plants using celite for mechanical injury. After 24 hours of extract inoculation, lower leaves will be inoculated with centrifuged sap obtained from virus infected indicator plant. Lesion will be observed after 7 days. The reduction of virus infection will be calculated to all treatments, using the following equation:

Inhibition % = A-B X 100/A

where:

A = Total number of systemic infected plants (untreated with botanicals + virus inoculated).

B = Total number systemic infected plants (treated with botanical sap + virus inoculated).

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